

**Platelet and endothelial function:
Polycystic ovary syndrome and the
renin-angiotensin system**

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Table of contents

List of figures	ix
List of tables	xi
Thesis summary	xii
Glossary of abbreviations	xxiii
Declaration	xxv
Acknowledgements	xxvi
Publications, presentations and awards	xxvii

CHAPTER 1

Platelet and endothelial function, metabolic syndrome and the renin angiotensin system

Overview	2
-----------------	----------

CHAPTER 1A

Abnormalities of platelet and endothelial function in cardiovascular diseases and cardiovascular risk states

Section A, Part 1: Normal platelet function

A1.1 Platelet anatomy	4
A1.2 Platelet adhesion	6
A1.2.1 von Willebrand Factor (vWf)	
A1.2.2 Adhesion receptors on platelets	
A1.3 Mechanism(s) of platelet activation	7
A1.3.1 Platelet activation by thrombin	
A1.3.2 Platelet activation by adenosine diphosphate (ADP)	
A1.3.3 Platelet activation by thromboxane A2 and arachidonic acid	
A1.4 Platelet shape change	11
A1.5 Platelet aggregation	12
A1.5.1 Glycoprotein IIb/IIIa	
A1.5.2 Platelet recognition sites on fibrinogen	
A1.6 Assessment of platelet aggregation	13
A1.6.1 Optical platelet aggregometry	
A1.6.2 Impedance platelet aggregometry	
A1.6.3 The rapid platelet function assay	
A1.6.4 PFA-100 in vitro platelet function analyzer	
A1.6.5 Measures of platelet activation	

<u>Section A, Part 2: Vascular endothelium: Interaction with platelet function</u>	17
A2.1 Normal endothelial function	17
A2.1.1 Interaction with platelet function	
A2.2 Assessment of endothelial function	21
A2.2.1 Invasive intra-coronary studies	
A2.2.2 Venous occlusion plethysmography	
A2.2.3 Flow-mediated vasodilatation	
A2.2.4 Pulse wave analysis (PWA)	
A2.2.5 Asymmetric NG,NG-dimethyl-L-arginine (ADMA)	
A2.3 Mechanism(s) of endothelial dysfunction	29
A2.3.1 Reduced NO half-life: Concept and significance of oxidative stress	
A2.3.2 Impaired synthesis of NO: Substrate and cofactor availability	
A2.3.3 Reduced sensitivity of NO	
A2.3.4 Reduced NOS expression	
A2.4 Clinical implications for coronary risk	37

CHAPTER 1B

Nitric oxide resistance, a vascular and platelet phenomenon **38**

Section B, Part 1: Nitric oxide **38**

B1.1 Synthesis and biochemistry	38
B1.2 Bioreactivity of nitric oxide	40
B1.3 Mechanisms of action of nitric oxide	41
B1.3.1 Vascular tone	
B1.3.2 Vascular smooth muscle proliferation	
B1.3.3 Leukocyte-endothelial interactions	

Section B, Part 2: The concept of nitric oxide resistance **43**

B2.1 The phenomenon of nitric oxide resistance	43
B2.2 Nitric oxide resistance at the vascular level	44
B2.2.1 Nitric oxide resistance and its relationship to endothelial dysfunction	
B2.3 Nitric oxide resistance at the platelet level	46
B2.3.1 Mechanism(s) of platelet NO resistance	
B2.3.2 Significance	

CHAPTER 1C

Metabolic syndrome/polycystic ovary syndrome: Relationship to cardiovascular diseases **48**

Section C, Part 1: Introduction **48**

Section C, Part 2: Metabolic syndrome **49**

C2.1 Pathogenesis 49	
C2.1.1 Obesity and inflammation	
C2.1.2 Insulin resistance	
C2.2 Diagnosis of metabolic syndrome	51
C2.3 Metabolic syndrome: Predictor of cardiovascular disease	52

<u>Section C, Part 3: Polycystic ovary syndrome</u>	53
C3.1 Polycystic ovary syndrome (PCOS): An introduction	53
C3.2 Relationship of PCOS to cardiovascular risk factors and cardiovascular disease	55
C3.3 Role of insulin resistance in PCOS	56
C3.4 Abnormalities in endothelial function in PCOS	57
C3.5 Polycystic ovary syndrome and the coagulation system	60
C3.6 Evidence for increased oxidative stress in polycystic ovary syndrome	61
C3.7 Subclinical cardiovascular disease in polycystic ovary syndrome	62
C3.8 Polycystic ovary syndrome and cardiovascular prognosis	64

CHAPTER 1D

The renin angiotensin system and platelet function: A work in progress	66
---	-----------

<u>Section D, Part 1: 'Classical' versus 'contemporary' renin angiotensin system, including ACE and ACE-2</u>	66
--	-----------

D1.1 Introduction	66
D1.2 Angiotensin-converting enzyme-2	68

<u>Section D, Part 2: Angiotensin II</u>	70
---	-----------

D2.1 Introduction	70
D2.2 Effects of Ang II on haemodynamic effects and cell growth	71
D2.3 Evidence for a role of Ang II in mediating oxidative stress	73
D2.4 Ang II: An atherogenic factor?	75
D2.5 Mediation of Ang II: Receptors and signal transduction	76

<u>Section D, Part 3: Angiotensin-(1-7)</u>	78
--	-----------

D3.1 Synthesis and catabolism	78
D3.2 Potential biological actions	79
D3.2.1 Actions in the heart and blood vessels	
D3.2.2 Actions as a growth tissue regulator	
D3.2.3 Actions in the kidneys	
D3.2.4 Actions in the central nervous system	
D3.3 Mediation of Ang-(1-7) actions: Receptors and signal transduction	84
D3.3.1 Binding to Ang-(1-7) receptor(s)	
D3.3.2 Binding to AT1/AT2 receptor	
D3.3.3 Binding to ACE	
D3.3.4 Interaction between Ang-(1-7) and bradykinin	
D3.3.5 Ang-(1-7): Release of prostanoids/nitric oxide	

<i>Section D, Part 4: Potential for therapeutic manipulation of the renin angiotensin system: Current and future options</i>	87
D4.1 Angiotensin-converting enzyme inhibitors	87
D4.2 Ang II AT₁ receptor antagonists	89
D4.3 Other therapeutic options: Aldosterone receptor blockers	92

<i>Section D, Part 5: Does the renin-angiotensin system impact on platelet function?</i>	93
---	-----------

CHAPTER 1E

Scope of currently described studies	95
---	-----------

CHAPTER 2

Materials and methods	96
------------------------------	-----------

2.1 Materials	96
2.1.1 Subjects studied	
2.1.2 Blood sampling	
2.1.3 Preparation of platelet-rich plasma	
2.2 Methods	97
2.2.1 Techniques for evaluation of platelet function	
2.2.2 Techniques for evaluation of endothelial function	
2.2.3 Other assays	

CHAPTER 3

Determination of platelet and endothelial function in polycystic ovary syndrome	104
--	------------

3.1 Summary of study examining platelet and endothelial function in women with polycystic ovary syndrome	104
3.1.1 Objectives	
3.1.2 Methods	
3.1.3 Results	
3.1.4 Conclusions	
3.2 Introduction	106
3.3 Objectives of the study	108
Primary	
Secondary	
3.4 Methods	109
3.4.1 Study population	
3.4.2 Study protocol	
3.4.3 Platelet aggregation studies	
3.4.4 Pulse wave analysis	
3.4.5 Definitions	
3.5 Data analysis	112

3.6	Results	113
3.6.1	Subject characteristics	
3.6.2	Platelet aggregation	
3.6.3	Platelet sensitivity to nitric oxide	
3.6.4	Relationship between aggregability and platelet responsiveness to nitric oxide	
3.6.5	Endothelium-dependent and -independent vasodilatation	
3.6.6	Biochemical markers	
3.7	Discussion	129
3.8	Study limitations	135
3.9	Conclusion	136

CHAPTER 4

	Effects of ramipril therapy on platelet responsiveness to nitric oxide	137
4.1	Overview	137
4.1.1	Objectives	
4.1.2	Methods	
4.1.3	Results	
4.1.4	Conclusions	
4.2	Introduction	139
4.3	Objectives of the study	141
	Primary	
	Secondary	
4.4	Methods	142
4.4.1	Study population	
4.4.2	Study protocol	
4.4.3	Criteria for withdrawal	
4.4.4	Endpoints	
4.4.5	Statistical analysis	
4.5	Results	147
4.5.1	Subject characteristics	
4.5.2	Platelet aggregation and inhibition with SNP	
4.5.3	Effects of ramipril in the presence of NO resistance	
4.5.4	ADP-induced aggregation in patient subgroups	
4.5.5	Augmentation index (AIX)	
4.5.6	Oxidative stress and endothelial function	
4.6	Discussion	153
4.7	Study limitations	155
4.8	Conclusions	156

Differential roles of angiotensin-(1-7) and angiotensin II on platelet function	157
5.1 Introduction	157
5.1 Study examining effects of Angiotensin-(1-7) and Angiotensin II on platelet aggregation and platelet responsiveness to the anti-aggregatory action of nitric oxide donor sodium nitroprusside	158
5.1.1 Summary	
5.1.2 Introduction	
5.1.3 Objectives of the study	
5.1.4 Methods	
5.1.5 Data analysis	
5.1.6 Results	
5.1.7 Discussion	
5.1.8 Limitation(s) of the study	
5.1.9 Conclusion	
5.2 Study examining the potential role of the Ang-(1-7) receptor in mediating the effects of Ang-(1-7) on platelet NO responsiveness and the relationship between Ang-(1-7) and oxidative stress	174
5.2.1 Summary	
5.2.2 Introduction	
5.2.3 Objectives of the study	
5.2.4 Methods	
5.2.5 Data analysis	
5.2.6 Results	
5.2.7 Discussion	
5.2.8 Limitation(s) of the study	
5.2.9 Conclusions	
5.3 Study examining whether the effects of exogenous Ang-(1-7) on platelet NO responsiveness are affected by the presence of ACE inhibitor therapy	188
5.3.1 Summary	
5.3.2 Introduction	
5.3.3 Objective	
5.3.4 Methods	
5.3.5 Data analysis	
5.3.6 Results	
5.3.7 Discussion	
5.3.8 Limitation(s) of the study	
5.3.9 Conclusions	

CHAPTER 6

Conclusions and future directions	199
6.1 Polycystic ovary syndrome	200
6.2 The renin-angiotensin system and platelet function	202
<hr/>	
References	205
<hr/>	
Appendices	227

List of figures

Figure 1.1	Mechanism(s) of platelet activation	8
Figure 1.2	Schematic overview of biochemical pathways related to ADMA Methylation of arginine residues within proteins or polypeptides occurs through N-methyltransferases, which utilize S-adenosylmethionine as a methyl group donor.	27
Figure 1.3	Factors that may contribute to a reduction in nitric oxide bioavailability.	30
Figure 1.4	A Venn diagram of reproductive phenotypes in PCOS: the definition of PCOS is variable depending on the union or intersection of phenotypes. PCOS is defined as a combination of signs and symptoms HCA (hyperandrogenic chronic anovulation); HPCO (hyperandrogenic polycystic ovaries); HCA-PCO (hyperandrogenic chronic anovulation with polycystic ovaries); PCO-CA (chronic anovulation with polycystic ovaries).	53
Figure 3.1	Extent of whole blood platelet aggregation induced by ADP L-PCOS and O-PCOS groups were hyperaggregable compared to normal subjects (2-way ANOVA, $P < 0.001$ for both *L-PCOS vs Normal and †O-PCOS vs Normal respectively).	116
Figure 3.2	Relationship between extent of platelet aggregation induced by ADP (2.5 $\mu\text{mol/L}$) and BMI: PCOS cohort.	117
Figure 3.3	Relationship between extent of platelet aggregation induced by ADP (2.5 $\mu\text{mol/L}$) and insulin resistance, determined by HOMA-IR: PCOS cohort.	117
Figure 3.4	Relationship between extent of platelet aggregation induced by ADP (2.5 $\mu\text{mol/L}$) and testosterone concentrations: PCOS cohort.	118
Figure 3.5	Inhibition of platelet aggregation by NO donor SNP.	119
Figure 3.6	Relationship between platelet NO responsiveness and BMI: PCOS cohort.	120
Figure 3.7	Relationship between platelet NO responsiveness and HOMA-IR, a measure of insulin resistance: PCOS cohort.	120
Figure 3.8	Relationship between platelet NO responsiveness and testosterone concentrations: PCOS cohort.	121
Figure 3.9	Platelet NO response versus ADP-induced aggregability (2.5 $\mu\text{mol/L}$) relationship: Comparison of normal and PCOS subjects.	122
Figure 3.10	Relationship between NTG response measured by AUC and baseline Aix: O-PCOS cohort. There was no significant relationship between baseline Aix and AUC (coefficient of determination, $r^2 = 0.019$, $p = 0.68$).	123
Figure 3.11	Endothelium-independent and -dependent responses.	124
Figure 3.12	Relationship between extent of aggregation, induced by ADP (2.5 $\mu\text{mol/L}$) and MDA concentrations: PCOS cohort.	126
Figure 3.13	Relationship between platelet NO responsiveness and MDA concentrations: PCOS cohort.	126
Figure 3.14	Mean ADMA and SDMA concentrations in PCOS subgroups compared with normals.	127
Figure 3.15	Mean hs-CRP concentrations in PCOS subgroups compared to normal cohort.	128
Figure 4.1	Trial schematic	145
Figure 4.2	Effects of 12 weeks of ramipril or placebo on the relationship between ADP and SNP responses (ANCOVA, $p < 0.001$)	149
Figure 4.3	Inhibition of aggregation after 12 weeks of ramipril or placebo in patients with 'normal' SNP responses (normal responders) versus patients with 'impaired' SNP responsiveness (NO resistant) at baseline	150
Figure 4.4	Effects of 12 weeks of ramipril or placebo on the relationship between ADP and SNP responses in patients with 'impaired' (upper graph, ANCOVA $p < 0.001$) vs 'normal' (lower graph, ANCOVA $p = \text{NS}$) responsiveness to SNP at baseline	151

Figure 5.1	Representative tracings for whole blood platelet aggregation.	163
Figure 5.2	Whole blood platelet aggregation induced by 1 $\mu\text{mol/L}$ U46619 (upper panel), and its inhibition by 10 $\mu\text{mol/L}$ SNP (lower panel).	165
Figure 5.3	Absolute differences (δ) in extent (upper panel) and rate (lower panel) of aggregation in the presence of SNP plus variable concentrations of Ang-(1-7) versus SNP alone, expressed as a percentage of U46619-induced aggregation.	167
Figure 5.4	Absolute differences (δ) in extent of aggregation in the presence of SNP plus variable concentrations of Ang-(1-7) versus SNP alone, expressed as a percentage of ADP-induced aggregation.	168
Figure 5.5	Absolute differences (δ) in extent (panels A and C) and rate (panels B and D) of U46619-induced platelet aggregation in the absence and presence of Ang II in variable concentrations (upper panels), and in the presence of SNP plus variable concentrations of Ang II versus SNP alone (lower panels).	169
Figure 5.6	Absolute differences (δ) in extent of aggregation in the presence of SNP plus variable concentrations of Ang-(1-7) and/or its antagonist, (D-Ala ⁷)-Ang-(1-7) versus ADP-induced aggregation alone.	181
Figure 5.7	Effect of Ang-(1-7) (1 nmol/L- 1 $\mu\text{mol/L}$) in the presence of SNP (1 $\mu\text{mol/L}$) versus SNP alone (black bar) on magnitude of ADP-induced aggregation in PRP.	181
Figure 5.8	Absolute differences (δ) in inhibition of aggregation in the presence of SNP plus variable concentrations of Ang-(1-7) (1 nmol/L- 1 $\mu\text{mol/L}$) versus SNP alone in platelet-rich plasma.	182
Figure 5.9	Absolute (δmV) and percentage (%) change in intensity of aggregation-induced LDCL by Ang-(1-7) (1 nmol/L- 1 $\mu\text{mol/L}$) in the presence of ADP (2.5 $\mu\text{mol/L}$) versus ADP-induced LDCL alone in whole blood (δmV of control; $\delta\%$ of control)	183
Figure 5.10	Percentage (%) change in intensity of aggregation-induced LDCL by Ang-(1-7) (1 nmol/L- 1 $\mu\text{mol/L}$) in the presence of SNP versus SNP alone. whole blood	183
Figure 5.11	Extent of ADP-induced aggregation in the ramipril and placebo groups at baseline and at 12 weeks	194
Figure 5.12	Extent of inhibition of ADP-induced aggregation by NO donor, SNP, in the ramipril and placebo groups at baseline and at 12 weeks	195
Figure 6.1	Functional balance between platelets and leukocytes	203

List of tables

Table 3.1	National Cholesterol Education Program's Adult Treatment Panel III report (ATP III) Clinical Identification of the Metabolic Syndrome (3 of the following 5)	111
Table 3.2	Baseline characteristics of all participants	113
Table 3.3	Baseline characteristics of subgroups	114
Table 3.4	Prevalence of the Metabolic Syndrome in the PCOS cohort	115
Table 3.5	Platelet aggregability in response to ADP	115
Table 3.6	Inhibition of platelet aggregation by NO donor SNP	118
Table 3.7	Biochemical markers	125
Table 4.1	Patient demographics at study entry: Clinical parameters and coronary risk factors	147
Table 4.2	Pharmacotherapy at baseline (%)	148
Table 4.3	Effect of ramipril therapy (12 weeks) on measured parameters	148
Table 4.4	Change in levels of biochemical markers in subjects who had normal or impaired SNP response at baseline with 12 weeks of ramipril or placebo	152
Table 5.1	Baseline characteristics of patients with ACS	161
Table 5.2	Baseline characteristics of all participants	193
Table 5.3	Endogenous angiotensin peptide levels at baseline and at 12 weeks	196

Thesis summary

Background

The phenomenon of platelet hyperaggregability and decreased platelet responsiveness to nitric oxide (also termed as nitric oxide resistance), documented in several cardiovascular disease states, is associated with adverse cardiovascular outcomes.

The series of experiments described in this thesis address primarily some aspects of the pathophysiology, epidemiology and therapy of the phenomenon of end-organ resistance to nitric oxide (NO) in two important conditions, that are closely associated with cardiovascular risk factors and disease states:-

- Polycystic ovary syndrome, which is closely linked with the metabolic syndrome and premature subclinical atherosclerosis.
- The renin-angiotensin system, which is recognized as a significant mediator in the pathophysiology of a number of cardiovascular disease states.

The first study examined the epidemiology/pathophysiology of putative platelet/endothelial dysfunction in young individuals with PCOS. The subsequent studies focused on the potential impact of the renin-angiotensin system on platelet and endothelial function. This mechanistic review is set in the context of a number of recent major clinical studies which have demonstrated surprising efficacy of certain angiotensin-converting enzyme (ACE) inhibitors (ramipril and perindopril) in the prevention of thrombotic processes. Thus we tested the hypothesis whether ACE inhibitor ramipril sensitizes platelets to NO (as a potential mechanism for improved cardiovascular outcomes) in a high risk patient cohort. In addition, particular attention will be given to the emerging role of the heptapeptide Angiotensin-(1-7), a possible physiological antagonist to Angiotensin II in the vasculature and the limitation of the current literature concerning potential effects of the renin-angiotensin system on thrombotic mechanisms.

Study examining platelet and endothelial function in women with polycystic ovary syndrome

Background

Polycystic ovary syndrome (PCOS) is frequently associated with obesity and the metabolic syndrome, which may imply incremental cardiovascular risk. The aim of this study was to determine whether PCOS is associated with platelet and/or vascular endothelial dysfunction, and whether this dysfunction is influenced by the presence of obesity and insulin resistance.

Methods and results

In this pilot study, women with PCOS (n=24; mean age 32 ±1) were evaluated in lean (L-PCOS; n=12) and obese (O-PCOS; n=12) subgroups, and compared with age-matched lean normal women (n=12). Platelet aggregation, its inhibition by nitric oxide (NO) donor sodium nitroprusside (SNP) and vascular endothelial function were assessed. Plasma concentrations of malondialdehyde (MDA), N^G , *NG*-dimethyl-L-arginine (ADMA) and high-sensitivity C-reactive protein (hs-CRP) were measured as markers of oxidative stress, endothelial dysfunction and inflammation respectively. Only one subject with PCOS met the Adult Treatment Panel III diagnostic criteria for metabolic syndrome. Platelet aggregation to adenosine diphosphate (ADP) 1 μM, 2.5 μM and 5.0 μM was 0.4±0.2 ohms, 5.4±0.8 ohms and 6.8±0.8 ohms respectively in the normal group. Comparatively, aggregation was significantly greater in both PCOS subgroups (p<0.001 for aggregation at all three concentrations of ADP in both L-PCOS and O-PCOS subgroups). Responses to NO inhibition of aggregation to ADP (2.5 μmol/L) were 40.0±7.5%, 38.4±6.4% and 72.4±6.0% in L-PCOS, O-PCOS and normal groups respectively (p<0.01 for both: L-PCOS vs normal and O-PCOS vs normal). Similar reduction in responses was seen at ADP 5 μmol/L in both PCOS subgroups. Endothelium-dependent vascular responses to salbutamol were significantly reduced in both PCOS subgroups compared to the normal cohort [peak responses (measured by peak change in augmentation index) was 1.2±2.1%, 1.9±1.6% and -5.2±1.7% in L-PCOS, O-PCOS and normal groups respectively, p<0.05 for both comparisons). Plasma concentrations of MDA were increased in PCOS subjects, independent of subgroup (0.25±0.02 μmol/L, 0.25±0.02 μmol/L and

0.17±0.02 µmol/L in L-PCOS, O-PCOS and normal groups respectively, $p < 0.05$ for both comparisons). ADMA ($p < 0.01$) and hs-CRP ($p < 0.02$) were raised in the PCOS group, primarily due to elevation in the O-PCOS group.

Conclusions and future implications

PCOS subjects, independent of obesity and insulin resistance, exhibit substantial platelet and vascular endothelial abnormalities, as well as evidence of incremental oxidative stress compared to age-matched healthy normal women. These anomalies may represent a significant basis for cardiovascular risk in women with PCOS, independent of metabolic syndrome. Detailed evaluation of the epidemiological and therapeutic implications of these anomalies is therefore of considerable priority. The future directions following these results will be as follows:

- To assess the evolution of these anomalies with advancing age.
- To evaluate the impact of treatment with oral contraceptives and insulin sensitizers, agents frequently used in the treatment of this patient group.
- To elucidate if these anomalies are accentuated with the presence of the metabolic syndrome

The subsequent studies evaluate the following hypothesis:

- Whether ACE inhibitors sensitize platelets to NO (as a potential mechanism for improved cardiovascular outcomes) in a high risk patient cohort.
- Whether Ang-(1-7) is a possible modulator of tissue responsiveness to NO, and thus of the phenomenon of NO resistance at the platelet level
- Whether the effect of Ang-(1-7) on platelet NO responsiveness is mediated by an Ang-(1-7) receptor and is platelet-mediated. In addition, the relationship between Ang-(1-7) and oxidative stress is explored.
- Whether the effect of Ang-(1-7) on platelet NO responsiveness is influenced by ACE inhibitor therapy.

Study examining the effects of ACE inhibitors on platelet aggregation and platelet responsiveness to the anti-aggregatory action of Nitric Oxide Donor Sodium Nitroprusside in a high risk patient cohort.

Background

ACEI have previously been shown to have beneficial effects in preventing ischemic coronary events; however, the mechanisms underlying these effects remain uncertain. We tested the hypotheses that (a) ramipril sensitizes platelets to NO in a high risk (HOPE study-type) patient cohort, and (b) that these putative effects of ramipril are more pronounced in patients with platelet NO resistance.

Methods and results

In a double-blind study, 119 patients who either had known ischemic heart disease or diabetes plus additional coronary risk factor(s) were randomized to receive ramipril (n=60; 10mg/day) or placebo (n=59). Whole blood platelet aggregation and its inhibition by the NO donor sodium nitroprusside (SNP) were assessed at baseline and 3 months after randomization. Responses to SNP were analyzed relative to ADP response during the study. Platelet NO resistance was defined as <35 % inhibition of aggregation by SNP. Plasma levels of asymmetric dimethyl arginine (ADMA) were measured as an index of endothelial dysfunction, while augmentation index [AIx] was assessed as a manifestation of arterial wave reflection. Mean patient age was 67±8 (SD) years. Ramipril therapy, while not affecting platelet aggregation, significantly increased SNP response relative to extent of aggregation in the entire cohort (p<0.001, ANCOVA). However, increases in unadjusted response to SNP occurred entirely in the NO-resistant patient subgroup (n=49; $\Delta 33.1 \pm 6.2$ vs $\Delta 13.5 \pm 5.1$ % increase for ramipril and placebo groups respectively, p=0.02). Ramipril also reduced AIx (p=0.02) and ADMA levels (p=0.05).

Conclusions

In a HOPE study-type patient population, ramipril sensitizes platelets to NO. This effect largely reflects amelioration of NO resistance in patients with impaired platelet NO response. Reversal of platelet NO resistance may contribute to the beneficial effects of ramipril in patients at high risk of ischemic events.

Study examining effects of Angiotensin-(1-7) and Angiotensin II on platelet aggregation and platelet responsiveness to the anti-aggregatory action of Nitric Oxide Donor Sodium Nitroprusside

Background

Patients with ischemic heart disease have platelets that are resistant to the anti-aggregatory effects of nitric oxide (NO) donors. This resistance is largely a result of accelerated NO clearance by superoxide radical (O_2^-). While Ang II has been shown to augment superoxide formation, recent studies have demonstrated that Ang-(1-7) has potentially opposite actions to those of Ang II in the vasculature. The current study compares the effects of Ang-(1-7) and Ang II on platelet aggregation and platelet responsiveness to the NO donor, SNP.

Methods and results

In whole blood samples obtained from normal subjects (n=17) and patients with acute coronary syndromes (ACS, n=17), platelet aggregation was induced by the thromboxane A_2 mimetic, U46619 (1-5 $\mu\text{mol/L}$), and ADP (2.5-5 $\mu\text{mol/L}$). SNP (10 $\mu\text{mol/L}$) was added 1 minute prior to the addition of agonist and its inhibitory effect on platelet aggregation was quantitated in the presence and absence of Ang-(1-7) (1 nmol/L-1 $\mu\text{mol/L}$). The effects of Ang II (1 nmol/L-1 $\mu\text{mol/L}$) were studied in a similar manner in 8 normal subjects. Ang-(1-7) did not significantly affect U46619- or ADP-induced platelet aggregation, but at concentrations of 10-100 nmol/L, potentiated the anti-aggregatory effects of SNP. With U46619 as an agonist, the maximum absolute potentiating effects were $25\pm 4\%$ and $23\pm 3\%$ for normal subjects and patients respectively ($p < 0.001$ for both). At higher concentrations (1 $\mu\text{mol/L}$), this effect was absent. Ang II (10-100 nmol/L) increased U46619-induced platelet aggregation ($p < 0.01$), irrespective of presence or absence of SNP.

Conclusions

Ang-(1-7) potentiates the effects of NO in inhibiting platelet aggregation, while Ang II potentiates the proaggregatory effects of thromboxane A₂ mimetic. Thus, these peptides exert biologically "opposing" effects in an indirect manner. It remains uncertain whether these effects, which occurred in vitro at supraphysiological concentrations of both angiotensin peptides, are representative of physiological antagonism.

Study examining the potential role of the Ang-(1-7) receptor in mediating the effects of Ang-(1-7) on platelet NO responsiveness and the relationship between Ang-(1-7) and oxidative stress

Background

Ang-(1-7) potentiates the effects of NO in inhibiting platelet aggregation. The mechanism(s) underlying this observation is unclear. This study is aimed at investigating the putative role of a specific Ang-(1-7) receptor in mediating this effect. In addition, potential relationships between Ang-(1-7) and platelets as well as oxidative stress are explored.

Methods and results

Platelet aggregation studies involving the use of Ang-(1-7) antagonist (Study 5.2A) and oxidative stress (Study 5.2C) were performed in whole blood. Platelet-rich plasma was utilized to assess whether the effect of Ang-(1-7) platelet NO responsiveness is mediated specifically by platelets (Study 5.2B). Ang-(1-7) exerted a concentration-dependent biphasic effect on platelet responses to the inhibitory actions of NO, with a potentiation of NO response at low concentrations (10nmol/L-100nmol/L) ($p < 0.01$) and abolition of effect at higher concentrations (1 μ mol/L). While pre-treatment with Ang-(1-7) antagonist, D-ala⁷-Ang-(1-7) (1 μ mol/L) alone did not exert any significant effects on platelet aggregation induced by ADP in the presence and absence of SNP, it completely inhibited the potentiation of Ang-(1-7) on NO response at low concentrations ($p < 0.01$). In platelet-rich plasma, Ang-(1-7) did not alter ADP-induced aggregation in the absence or presence of SNP. As regards Study 5.2C, Ang-(1-7) did not significantly alter the intensity of aggregation-induced lucigenin-derived chemiluminescence (LDCL) by ADP (2.5 μ mol/L) in whole blood. However, Ang-(1-7) at low concentrations (1nmol/L) significantly ($p = 0.024$) potentiated NO inhibition of aggregation-induced LDCL. At higher concentrations (1 μ mol/L), there was a non-significant trend towards an opposite effect of Ang-(1-7) on NO inhibition of aggregation-induced LDCL.

Conclusions

Firstly, Ang-(1-7) at low concentrations mediates the potentiation of the anti-aggregatory effects of NO via a specific Ang-(1-7) receptor. Secondly, Ang-(1-7) potentiation of platelet NO responsiveness is not directly mediated by platelets. Thirdly, Ang-(1-7), at low concentrations, significantly attenuates

aggregation-induced superoxide production in whole blood. This study provides the first evidence for the existence of a specific Ang-(1-7) receptor in human whole blood as well as evidence for a relationship between Ang-(1-7) and oxidative stress.

Study examining whether the effects of exogenous Ang-(1-7) on platelet NO responsiveness are affected by the presence of ACE inhibitor therapy

Background

While studies have shown that exogenous Ang-(1-7) has effects on both vascular as well as platelet function, there is little evidence of its benefit in the presence of chronic ACE inhibition therapy in humans. The objective of this study was to evaluate whether supraphysiological concentrations of Ang-(1-7) exert a differential effect on platelet function in the presence of chronic ACE inhibition.

Methods and results

A subset of patients (n=44) who are at high cardiovascular risk (enrolled in the study reported in Chapter 4) were randomized to ramipril (10 mg) (n=22) and placebo (n=22). Platelet aggregation studies were performed in whole blood samples using ADP (2.5 $\mu\text{mol/L}$) as an agonist. SNP (10 $\mu\text{mol/L}$) was added 1 minute prior to the addition of agonist and its inhibitory effect was quantitated in the presence and absence of Ang-(1-7) (1 nmol/L-1 $\mu\text{mol/L}$). Twelve weeks of ramipril therapy did not significantly alter ADP-induced aggregation or platelet responsiveness to NO in this patient population. Ramipril therapy significantly increased endogenous Ang-(1-7) levels and decreased Ang II/ Ang I ratio. Following 12 weeks of therapy compared to baseline, there was no significant change in the effect of Ang-(1-7) on platelet NO responsiveness within each group ($p=0.15$) and between each group ($p=0.60$) by 2-way ANOVA.


Conclusions

This study has found no evidence that chronic treatment with the ACE inhibitor ramipril affects the interaction between Ang-(1-7) and platelet responsiveness to NO.

The future directions following these results will be as follows:-

- To examine the physiological significance of Ang-(1-7) on platelet function possibly by administration of a specific Ang-(1-7) antagonist in vivo.
- To explore strategies that could result in incremental concentrations of Ang-(1-7).
- To study further the physiological mechanisms/significance of platelet-leukocyte interactions and Ang-(1-7).

Glossary of abbreviations

Abbreviation	Definition
AA	Arachidonic acid
ACS	Acute coronary syndrome
ADMA	Asymmetric N ^G ,N ^G -dimethyl-L-arginine
ADP	Adenosine diphosphate
AIx	Augmentation index
ANCOVA	Analysis of covariance
Ang	Angiotensin
ANOVA	One-way analysis of variance
ATP III	National Cholesterol Education Program's Adult Treatment Panel III
AUC	Area under curve
BK	Bradykinin
BMI	Body mass index
C	Celsius
Ca ²⁺	Calcium
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CVD	Cardiovascular disease
DAG	Diacylglycerol
DDAH	dimethylarginine dimethylaminohydrolase
EDTA	Ethylenediamine tetraacetic acid
eNOS	Endothelial nitric oxide synthase
FAD	flavin adenine dinucleotide
FBF	Forearm blood flow
FMD	Flow-mediated vasodilatation
FMN	flavin mononucleotide
Gamma	
GP	Glycoprotein
H ₂ O ₂	Hydrogen peroxide
HDL	High-density lipoprotein
HOMA-IR	Homeostasis model of insulin resistance
hs-CRP	high-sensitivity C-reactive protein
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IP ₃	Inositol 1,4,5-trisphosphate
ISDN	Isosorbide dinitrate
LDL	Low-density lipoprotein

L-NMMA	N ^G -monomethyl-L-arginine
L-PCOS	Lean-PCOS
MDA	malondialdehyde
NADPH	nicotinamide adenine dinucleotide phosphate
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NO ⁻	Nitroxyl
NO ⁺	Nitrosonium
NOS	Nitric oxide synthase
GTN	Nitroglycerin
O-PCOS	Obese-PCOS
PAI-1	plasminogen activator-1
PCOS	Polycystic ovary syndrome
PG	Prostaglandin
PIP ₂	phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PLA ₂	Phospholipase A ₂
PLC	Phospholipase C
PPP	Platelet-poor plasma
PRP	Platelet-rich plasma
PWA	Pulse wave analysis
QUICKI	Quantitative insulin sensitivity check index
ROS	Reactive oxygen species
SAP	Stable angina pectoris
SDMA	N ^G ,N ^G -dimethyl-L-arginine
SEM	Standard error of mean
sGC	Soluble guanylate cyclase
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
Superoxide	O ₂ ⁻
TBA	Thiobarbiturate acid
TNF α	Tumour necrosis factor α
TxA ₂	Thromboxane A ₂
TxR	Thromboxane A ₂ receptor
VASP	Vasodilator-stimulated phosphoprotein
VSMCs	Vascular smooth muscle cells
vWf	Von Willebrand factor

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, the thesis contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the university library, being available for loan and photocopying.

Sharmalar Rajendran

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Publications, presentations and awards

Peer reviewed articles relating to this thesis

Rajendran S, Chirkov YY, Campbell DJ, Horowitz JD. Angiotensin-(1-7) Enhances Anti-Aggregatory Effects of the Nitric Oxide Donor Sodium Nitroprusside. *J Cardiovasc Pharmacol* 2005; 46: 459-463

Rajendran S, Chirkov YY and Horowitz JD. Potentiation of platelet responsiveness to nitric oxide by angiotensin-(1-7) is associated with suppression of superoxide release. *Platelets*. 2007 Mar;18(2):158-64.

Accepted presentations at international meetings

S Rajendran, SR Willoughby, EA Kelly, T Heresztyn, R Norman and JD Horowitz. Severe platelet and endothelial dysfunction in women with polycystic ovary syndrome. Accepted for presentation at AHA scientific sessions 2005. (Nominated for Samuel A. Levine Young Investigator Award)

S Rajendran, YY Chirkov, DJ Campbell and JD Horowitz. Differential effects of Angiotensin II and Angiotensin-(1-7) on platelet aggregation. Accepted for presentation in Cardiac Society, CSANZ 2005

Yuliy Chirkov, Sharmalar Rajendran, Aaron Sverdlov, John D Horowitz. Potentiation of platelet responsiveness to nitric oxide by angiotensin-(1-7) is mediated by its specific receptor and is associated with the suppression of superoxide release. Accepted for presentation at ESC Congress, 2007

Scott R Willoughby, Sharmalar Rajendran, Elizabeth A Kelly, Tamila Heresztyn, Sue Leslie, Yuliy Y Chirkov and John D Horowitz.. Ramipril potentiates platelets nitric oxide responsiveness in patients with high cardiovascular risk. Accepted for presentation at ESC Congress, 2007

SR Willoughby, S Rajendran, EA Kelly, S Leslie, T Heresztyn, M Dronavalli, YY Chirkov, JD Horowitz. Hypertension is associated with impairment of platelet responsiveness to nitric oxide in a HOPE-type patient cohort. Accepted for presentation in Cardiac Society, CSANZ 2005

YY Chirkov, S Rajendran, JD Horowitz. Angiotensin-(1-7) improves platelet responsiveness to anti-aggregatory effects of nitric oxide. *European Heart Journal*, ESC Congress 2004

YY Chirkov, S Rajendran, JD Horowitz. Angiotensin-(1-7) potentiates the anti-aggregatory effects of nitric oxide. Accepted for presentation in AHA scientific sessions 2004.

Awards/grants relating to this thesis

University of Adelaide Medical Postgraduate Scholarship

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